Selected immunohistochemical features of conventional renal cell carcinomas coexpressing P53 and MDM2

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Renal clear cell carcinoma (CCRCC) is an aggressive tumor for which new prognostic factors are needed. It has been suggested that CCRCCs co-expressing P53 and MDM2 could represent a special subgroup; therefore the aim of this study was to explore their immunohistochemical features. The material studied consisted of 470 cases of CCRCC. Immunohistochemistry for MDM2, P53, Ki-67, VEGF-A, VEGF-C, VEGF-D, GLUT1, CA9, and CK 7 was performed on tissue microarrays and assessed semi-quantitatively. On average, 6.6% or 5.3% of cases were P53+/MDM2+, depending on the P53 antibody used. The mean percentage of Ki-67 positive cells was 0.6% and p53-positive MDM2-positive cases showed significantly higher expression of Ki-67. The other immunohistochemical parameters studied did not differ between p53-positive MDM2-positive cases and the rest of the subtypes studied. Expression of almost all immunohistochemical markers differed with respect to pT stage; only for CA9 was the difference not significant. Furthermore, almost all immunohistochemical markers studied differed with respect to differences in grade; only for GLUT1 was the difference not significant. Our results suggest that with the exception of Ki-67, there are no significant associations between analyzed markers and the double P53+/MDM2+ phenotype.

Key words: renal cell carcinoma, MDM2, P53, Ki-67, VEGF-A, VEGF-C, VEGF-D, GLUT1, CK 7, CA9.

Introduction

Renal cell carcinoma (RCC) constitutes approximately 9% of all human cancers. The most frequent variant, conventional or clear cell (CCRCC), is particularly interesting because of the considerable progress obtained in the last few years and the introduction of targeted therapy. In CCRCC patients, the prognosis is dependent on stage, grade and patient clinical condition. Other prognostic markers, including immunohistochemistry, have been studied; however, none have been introduced into clinical practice. It has been suggested that co-expression of P53 and MDM2 may define a distinct group of CCRCCs [1]. In our previous work [2] we explored this hypothesis by comparing the histologic prognostic factors such as stage, grade or presence of necrosis in tumors with or without doubly-expressed P53 and MDM2. The aim of the current study is to analyze the expression of potential immunohistochemical markers including Ki-67, VEGF-A, VEGF-C, VEGF-D, GLUT1, CK 7 and CA9 in samples expressing both P53 and MDM2, and to determine whether these immunohistochemical markers have prognostic significance.

Table I. Antibodies used in this study

Specificity	Dilution	Manufacturer	Clone/type
MDM2	1 : 50	Novocastra	1B10
P53	1 : 200	DAKO	DO-7
P53	1 : 50	Novocastra	PAb1801
Ki-67	1 : 50	DAKO	MIB-1
VEGF-A	1 : 100	Santa Cruz	polyclonal A20
VEGF-C	1 : 100	Santa Cruz	polyclonal H-190
VEGF-D	1 : 200	R&D systems	78923
GLUT1	1 : 50	DAKO	polyclonal MYM
CK 7	1 : 50	DAKO	OV-TL 12/30
CA9	1 : 100	Novocastra	TH22

Material and methods

The material analyzed was retrieved from the archives of the Department of Pathomorphology. Cases were reviewed by an expert urologic pathologist and reclassified according to the most recent WHO classification [3]. For the present study, only unequivocal conventional (clear cell) carcinomas were chosen. The tumors were graded according to the International Society of Urological Pathologists, which is derived from a modification of the Fuhrman method [4]; this is referred to as the Fuhrman method from this point onward. The presence of sarcomatoid components and necrosis was noticed, and in accordance with Delahunt *et al.* [5], another tumor grade was assigned that took necrosis into consideration.

For each case, a slide containing well preserved and representative tumor tissue was selected and a respective area was marked for study. Corresponding blocks were used to construct a tissue microarray (TMA) using Tissue MicroArrayer MTA-1 (Beecher Instruments Inc., Sun Prairie, USA). From each donor block, three 0.6 mm cylinders were selected. The acceptor paraffin blocks were prepared noting the location of each cylinder, and 3 μm-thick sections were cut.

Table II. Immunohistochemistry results

	Mean	Median	Range	SD
VEGF-A	1.12	1.00	0-3.00	0.82
VEGF-C	0.76	0.67	0-2.67	0.66
VEGF-D	0.30	0.00	0-3.00	0.48
GLUT1	0.41	0.17	0-2.33	0.53
CK7	0.16	0.00	0-1.00	0.33
CA9	1.88	2.00	0-3.00	0.96

For immunohistochemistry, a standard staining protocol was used. Briefly, the slides were dewaxed, rehydrated and incubated in 3% peroxide solution for 10 minutes to block endogenous peroxidase activity. Antigen retrieval was carried out by microwaving in citrate buffer (0.2% citric acid titrated to pH 6.0 with 2N NaOH) 3 times for 5 minutes each at 750 W. The primary antibodies are listed in Table I. The Lab Vision detection system (Thermo Fisher Scientific, Waltham, USA) was used. The chromogen used was 3-amino-9-ethylcarbazole. The slides were counterstained with Mayer hematoxylin (Thermo Fisher Scientific, Waltham, USA) and coverslipped. The immunohistochemistry was scored by one of the authors (M.H.) without knowledge of the clinicopathologic parameters and the results of scoring introduced into an Excel spreadsheet (Microsoft Corp., Redmond, USA). Samples were classified as positive if they expressed both P53 and MDM2, and if not, they were marked as negative. Ki-67 expression was quantified based on the percentage of immunopositive cells. Furthermore, to determine expression of each of CA9, CK 7, CD10, VEGF-A, VEGF-C, and VEGF-D, a scale from 0 to 3 was used, where 0 represented no staining and 3 represented strong staining of all cells. The results were averaged across all the scores available for the given case.

Cases lost from the TMAs were excluded from the study. Student's t, χ^2 and ANOVA tests were used where appropriate. Correlations were measured by Pearson's and gamma correlation coefficients. The statistical analysis was done with Statistica 10 PL (StatSoft Inc., Tulsa, USA) and P values less than 0.05 were considered significant.

Results

The material studied was obtained from 470 cases. There were 280 (59.6%) males and 190 (40.4%) females. The mean age of the patients was 61.3 years

Table III. Relationship between the immunohistochemistry results and pT stage

	VEGF-A		VEGF-C		VEGF-D		GLUT1		CK-7		CA9	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
pT1	1.00	0.79	0.69	0.60	0.22	0.40	0.32	0.45	0.23	0.39	2.00	0.95
pT2	1.17	0.87	1.01	0.76	0.45	0.68	0.38	0.46	0.18	0.31	1.98	1.01
pT3	1.23	0.83	0.78	0.69	0.35	0.50	0.50	0.59	0.10	0.27	1.78	0.95
pT4	0.67	0.88	0.44	0.19	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00
p	< 0.03		< 0.04		< 0.01		< 0.002		< 0.002		NS	

(range 26 to 92; SD 10.59). P53 expression, when staining with the PAb1081 antibody, was positive in 62 cases (13.2%). Furthermore, when using the p53-specific antibody, DO-7, expression was observed in 71 cases (15.1%). Reaction for MDM2 was positive in 178 cases (37.9%). There were 31 cases (6.6%) positive for both P53 and MDM2 using the DO-7 antibody, and 25 cases (5.3%) when using the PAb1081 antibody. Further details with respect to the histopathologic characteristics and their relationship with P53 and MDM2 expression may be found in our previous work [2].

The mean percentage of Ki-67 positive cells was 0.6% (range 0 to 33% SD 2.6). P53-positive cases showed significantly higher Ki-67 expression (0.24 vs. 2.69%, $p << 0.01$ for DO-7 and 0.34 vs. 2.46%, $p << 0.01$ for PAb1081), yet the values for MDM2 positive and negative cases were only slightly different (0.52 vs. 0.66%, NS). Consequently, p53-positive MDM2-positive cases showed significantly higher expression (0.54 vs. 1.51%, $p < 0.05$ for DO-7 and 0.44 vs. 1.97%, $p < 0.02$ for PAb1081).

The results obtained when using other immunohistochemical stains are shown in Table II. Briefly, the tumors with sarcomatoid components showed lower expression of VEGF-A (1.15 vs. 0.69; $p < 0.004$) whereas tumors with detectable necrosis showed lower expression of VEGF-C (0.71 vs. 0.92; $p < 0.005$) but higher VEGF-D (0.28 vs. 0.39; $p < 0.04$). Somewhat surprisingly, expression of CA9 was lower in tumors showing necrosis (1.98 vs. 1.45; $p << 0.001$). Almost all immunohistochemical results differed when considering differences in pT stage, with CA-9 proving to be the only non-significant observation (Table III). Tumor diameter showed a weak correlation with immunohistochemical marker expression (Table IV). Almost all immunohistochemical data differed when tumor grade was also considered (evaluation by both the Fuhrman and Delahunt methods was undertaken; see Tables V and VI). However, the difference observed when quantifying GLUT1 expression was not significant, although close to significance when graded according to Fuhrman.

Table IV. Correlations between immunohistochemistry and tumor diameter

	DIAMETER
VEGF-A	$r = 0.1389$, $p = 0.012$
VEGF-C	$r = 0.0803$, $p = 0.147$
VEGF-D	$r = 0.0983$, $p = 0.075$
GLUT1	$r = 0.1106$, $p = 0.045$
CK7	$r = -0.1226$, $p = 0.026$
CA9	$r = -0.1721$, $p = 0.002$

There was a significant difference in VEGF-A expression between P53 negative and positive cases (for DO-7 stain 1.17 vs. 0.86; for PAb1081 1.17 vs. 0.87; both $p < 0.001$) as well as MDM2 negative and positive cases (1.05 vs. 1.25; $p < 0.001$). P53 negative and positive cases evaluated using the DO-7 antibody differed also in their CK7 expression (0.14 vs. 0.23; $p < 0.05$). When cases were evaluated using the PAb1081 antibody, P53 negative and

Table V. Relationship between the immunohistochemistry results and grading by the standard method

	VEGF-A		VEGF-C		VEGF-D		GLUT1		CK-7		CA9	
GRADE	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
1	0.83	0.70	0.53	0.53	0.15	0.30	0.35	0.54	0.23	0.37	2.19	0.88
2	1.16	0.76	0.75	0.68	0.29	0.46	0.36	0.49	0.14	0.32	1.87	0.97
3	1.59	0.86	1.06	0.70	0.50	0.60	0.50	0.55	0.10	0.28	1.48	0.91
4	0.78	0.73	0.77	0.61	0.35	0.54	0.49	0.56	0.15	0.32	1.83	1.03
p	<< 0.001		<< 0.001		<< 0.001		< 0.07 (N.S.)		< 0.03		<< 0.001	

Table VI. Relationship between the immunohistochemistry results and grading by the Delahunt [5] method

	VEGF-A		VEGF-C		VEGF-D		GLUT1		CK-7		CA9	
Grade	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
1	0.98	0.74	0.63	0.61	0.22	0.39	0.37	0.52	0.19	0.35	2.06	0.92
2	1.50	0.88	0.99	0.73	0.43	0.59	0.41	0.48	0.10	0.28	1.55	0.96
3	1.52	0.84	1.05	0.66	0.46	0.58	0.53	0.58	0.08	0.23	1.22	0.83
4	0.71	0.73	0.78	0.65	0.37	0.55	0.50	0.56	0.18	0.34	1.99	0.98
p	<< 0.001		<< 0.001		< 0.001		NS		< 0.05		<< 0.001	

positive cases differed in VEGF-C expression (0.73 vs. 0.97; $p < 0.01$). The immunohistochemical parameters studied did not differ significantly between p53-positive MDM2-positive cases and the rest of the cases studied; however, Ki-67 was an exception (see above).

Discussion

Renal cell carcinoma constitutes just 9% of human cancers [6], but is of interest to both the urologist and the urologic pathologist, due to the fact that it is the most aggressive of urologic malignancies. Furthermore, significant progress has been made of late both in understanding its biology and improving treatment. Prognostication in RCC is difficult and the best established morphologic prognostic factors include tumor type and stage, presence of sarcomatoid components, and for some subtypes, histologic grade [7]. Additional prognostic biomarkers would be highly clinically useful; however, to date, there are only limited data available; therefore further studies are needed before they might be used in clinical practice [7, 8].

The *TP53* gene is mutated in many human cancers, and this mutation paradoxically results in P53 protein accumulation. A significant proportion of RCC cases express the P53 protein product, yet the *TP53* mutation rate in RCC is low, suggesting another, poorly understood mechanism for protein accumulation [9]. The *MDM2* gene product participates in the same pathway as *TP53*; it is the main regulator of *TP53* functions, and its expression is reciprocally controlled by a P53-dependent mechanism. It has been hypothesized that RCCs expressing both P53 and MDM2 behave in a more aggressive fashion [1, 10, 11]. We decided to further explore this idea and in our previous work [2] we found a number of morphologic differences between P53/MDM2-positive and -negative CCRCC, which could influence the prognosis of this subgroup of tumors.

The basic carcinogenic mechanism in CCRCC involves abnormal activation of the so-called "hypoxia pathway". Furthermore, the most frequent cytogenetic event, a 3p deletion, leads to loss of the *VHL* gene. The *VHL* protein product is responsible for inactivating hypoxia inducible factor (HIF). Both in response to hypoxia and the loss of the *VHL* gene, HIF begins to fulfill its role as a transcription factor, leading to the expression of a number of genes. The protein products of these genes participate in both protecting the cell against hypoxia and activating angiogenesis. Among the genes in question there are carbonic anhydrase 9, vascular endothelial growth factors and their receptors, glucose transporter 1 and others [12-16].

Carbonic anhydrase 9 (CA9), like the other carbonic anhydrases, catalyzes the reversible hydration of carbon dioxide. This enzyme is not expressed in non-neoplastic kidney cells, and in several cancers CA9 has been shown to be a marker of hypoxia. Liao *et al*. [17] described the immunohistochemistry for CA9 as highly specific for CCRCC, and since then it has been used for diagnostic purposes. Genega *et al.* [18] studied CA9 expression in a large, heterogeneous group of renal tumors and confirmed that its expression is significantly more common in CCRCC, and in this tumor type is associated with the histologic grade. Sandlund *et al.* [19] analyzed the CA9 expression in different renal carcinomas, confirming its relative specificity for CCRCC. They failed to find a correlation between CA9 expression, tumor stage and grade, yet observed that tumors with low CA9 expression behave in a considerably more aggressive fashion and this effect was evident also in multivariate analysis. On the other hand, some of the most recent papers have failed to show the prognostic significance of CA9 [20, 21]. Zerati *et al.* [20] analyzed a group of non-metastatic RCCs, and found no relationship between CA9 and overall survival, tumor stage, size, invasiveness or vascular invasion. Zhang *et al.* [21] analyzed a large cohort of RCC cases with periods of observation exceeding 10 years. They found that low CA9 expression is predictive of poor prognosis in univariate analysis, both in terms of survival and time to metastasis, yet this effect disappeared when adjusted for nuclear grade and necrosis. The discordance in these results has been explained by comparing differences among the different groups of patients analyzed (i.e. entire CCRCC population vs. metastatic cases), differences in the size of the populations under

study or which technology was used (i.e. TMAs vs. whole sections). In our study, we observed that CA9 expression was inversely associated with tumor size, stage and grade. The lower CA9 expression in the cases where necrosis was evident may be due to the fact that CA9 regulation in CCRCC is not related to hypoxia itself, but to autonomous activation of the hypoxia pathway, characteristic for this cancer. Lastly, CA9 has also been proposed as a predictive marker in CCRCC. Atkins *et al.* [22] analyzed the influence of CA9 expression on immunologic treatment and concluded that only patients with tumors expressing high CA9 levels could benefit with prolonged survival after treatment with interleukin-2. More recently, Choueiri *et al.* [23] analyzed a group of metastasizing RCCs for CA9 expression. They concluded that although CA9 is not a prognostic factor in this particular subgroup of patients, it may serve as a predictor of treatment results with anti-angiogenic drugs.

Different vascular endothelial growth factors (VEGFs) participate in the development of blood vessels and lymphatic vessels. Of these, VEGF-A is the main regulator of blood vessel growth and differentiation. In CCRCC it is thought to act, in concert with its receptor, as the main autocrine stimulating loop for cancer cells. Following the understanding of this fact, antiangiogenic treatment for advanced CCRCC was developed. Furthermore, VEGF-A and its receptor VEGFR1 are the most important of the VEGF family when considering molecular pathogenesis in RCC. Accordingly, Gunningham *et al.* [24] failed to show upregulation of VEGF-C mRNA in CCRCC. They also observed no increase of VEGFR2 or VEGFR3, but reported a significant correlation between levels of VEGF-C and VEGFR3. In accordance with our previous discussion above, Lakovlev *et al.* [25] found a positive correlation between VEGF-A expression and tumor grade, when adjusted for the expression in normal renal medullary tissue. Furthermore, they also found an inverse association between microvascular density and survival, when adjusted similarly for expression in normal renal medullary tissue. Jacobsen *et al.* [26] observed a correlation between VEGF-A expression and survival, but only when analyzed with a univariate model. Baldewijns *et al.* [27] showed that CCRCC has only limited lymphangiogenic potential, and in fact were able to show that the density of lymphatic vessels was lower in cancer tissue when compared with non-neoplastic kidney tissue. Likewise, while the VEGF-A and C mRNA level were higher in cancer samples, VEGF-D mRNA level was higher in normal tissue. Interestingly, VEGF-D expression was quite low in comparison with other VEGF types in the samples studied during our investigation. Klatte *et al*. constructed a molecular prognostic model that included expression of Ki-67, VEGFR-1, VEGF-D and p53. This model showed higher power in predicting disease-free survival than standard systems, and when incorporating the standard clinicopathological data, correctly predicted the outcome in over 90% of cases [8].

Glucose transporter 1 (GLUT1) facilitates glucose transport across the cellular membrane, and is responsible for basic intracellular glucose uptake [28]. It has been shown that hypoxia induces GLUT1 expression. Abnormal HIF activation, such as the one seen in CCRCC, may cause the same effect [12]. Lidgren *et al*. [29] have shown that most cases of CCRCC express high levels of GLUT1. Interestingly, in contrast to papillary RCC, they found no correlation between GLUT1 expression and tumor stage. This contrasts with data obtained in the present study in that we observed a weak, yet significant, correlation between this marker and tumor diameter. Lidgren *et al.* observed low GLUT1 expression and determined that it was a favorable prognostic indicator both in papillary and clear cell RCC. Another interesting relationship was reported by Singer *et al.* [30], who found an inverse correlation between GLUT1 expression levels and effector T-lymphocyte activity. Therefore, hypoxia itself or activation of the hypoxia pathway might contribute to tumor-permitting immunosuppression.

Cytokeratin 7 (CK7) has a firmly established role as a diagnostic marker in renal tumor histopathology, especially because of its distinct staining pattern which is characteristic for chromophobe carcinoma [13, 31]. Only a subset (10-15%) of CCRCCs express CK7, and according to some reports, this expression may have an influence on prognosis. Mertz *et al.* [32] analyzed the expression of both CK7 and CK19. The CK7 and CK19 positive tumors tended to show fewer cytogenetic alterations and patients had a better prognosis. The significance of this observation was also noted when evaluating CK7 expression using multivariate analysis. Likewise, in our material, CK7 expression was seen in lower stage, lower grade tumors.

As a proliferative marker, Ki-67 appears to be an obvious prognostic factor, yet several studies have failed to confirm this in multivariate models. In the study by Kramer *et al.*, [33] Ki-67 expression was found to have an influence on survival, but this was seen only when univariate analysis was employed, whereas in multivariate models, the stage was the only variable of prognostic importance. The cited study analyzed a relatively small group of cases; and further, the material studied contained samples that were not only from clear cell carcinomas, but also other subtypes, which may have influenced the results. The results obtained by Cheville *et al.* [34] were more conclusive in that they analyzed a uniform group of pT1 CCRCCs and found that Ki-67 expression was significantly higher in the tumors that proved fatal, but the difference disappeared when ad-

justed for stage, grade and presence of necrosis. In contrast, Tollefson *et al.* [35] specifically addressed the relationship between Ki-67 expression and necrosis, and came to the conclusion that although a correlation between the two was present, they both contributed more strongly as independent read-outs of prognosis. The prognostic significance was also apparent when these data were evaluated using a multivariate model which took into account stage, grade and clinicopathologic parameters. Kankuri *et al.* [36] reported that tumors expressing both P53 and Ki-67 showed higher frequency of metastases. They also observed, similar to our study, an association between P53 and Ki-67 expression. On the other hand, Moch *et al*. reported that Ki-67 expression appeared to be related to survival; however, when evaluated using a multivariate model, only the influence of P53 and stage remained significant [37].

In conclusion, we have analyzed a number of potential prognostic markers in the context of P53 and MDM2 double expression in renal clear cell carcinoma. Our results suggest that, with the exception of Ki-67, there are no significant associations between the markers evaluated and P53+/MDM2+ phenotype.

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